Can Carbon Nanotubes Deliver on their Promise in Biology? Harnessing Unique Properties for Unparalleled Applications

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Introduction

Carbon nanotubes (CNTs) are rolled-up sheets of hexagonally ordered carbon atoms, giving tubes with diameters on the order of a few nanometers and lengths typically in the micron range. They may be single- or multi-walled (SWCNTs and MWCNTs respectively), and can be electrically conducting or semiconducting depending upon the orientation of the carbon lattice with respect to the tube axis (known as chirality in this context). Since the seminal report of their synthesis in 1991,[1] CNTs have fascinated scientists of all strides. Physicists have been intrigued by their electrical, thermal, and vibrational potential. Materials scientists worked on integrating them into ultra-strong composites and electronic devices, while chemists were fascinated by the effects of rolling on the conventionally planar hexagonal carbon lattice and developed new synthesis and purification techniques. Those in the life sciences considered how these engineered nanomaterials could bring radically new functions to biological systems and applications in medicine.

Now that the crown of “wonder material” in the public perception is being usurped by graphene, it is time to assess the real impact of CNTs. In terms of materials applications the results are clear – a bicycle made of a CNT-carbon fibre composite won the Tour de France in 2006,[2] and in 2013 a computer made from CNT-based transistors was unveiled.[3] But what is the status of their contribution to biological and medicinal applications? CNTs are certainly an enticing prospect in this area: their aspect ratio is thought to be responsible for their excellent cell penetration properties; anisotropic conductivity/semi-conductivity along their axis is ideal for integration with nervous and muscular tissue; an ultra-high surface area maximises their ability to ‘talk’ with biological matter; the hollow interior provides a very large cargo-carrying capacity for drug delivery; and their exteriors are readily functionalised to permit tailoring of solubility and biological recognition.

However, to date no large-scale, real-life biotechnological CNT breakthrough has been industrially adopted.[4] This is to a large extent due to a number of significant hurdles which CNTs present for the life sciences. The most significant issue is multiparametric sample variability in terms of length, diameter, number of walls, chirality, degree and arrangement of functionalization, and topological, graphitic, and residual catalyst impurities. These levels of variability can be minimised,[5] but not eliminated, and that only with significant sample loss and increasing financial burden. CNT polydispersity is not just a problem for repeatable and synthesis characterisation, but is also a major barrier to governmental approval of medicines, being much more complex than the comparatively well-defined length variation of

polymers used in some drug formulations (where a linear polydispersity index of 1.10 may be acceptable[6]) or the range of attachment number in antibody-drug conjugates.[7] Furthermore, while many groups have reported improved cell penetration with using CNTs, the exact mechanisms for uptake are still very much a matter of debate.[8] Recent toxicity studies have corrected some early misapprehensions and indicated that comparatively short functionalised CNTs are harmless and cleared easily, although these must be taken with the caveat of potential batch variability.[9] Bearing these considerations in mind, it is important to note that neither of the two current clinical trials involving CNTs have direct contact between CNTs and living biological matter, being externally-based diagnostic systems.[10]

A viable carbon nanotube biotechnology is one in which the unique properties of nanotubes bring about an effect that would be otherwise impossible.

What then should we make of the investment in resource which has been placed into biological applications of CNTs? The literature is replete with the appendage of CNTs to nearly every biological system available, but in the vast majority of cases the CNT provides an incremental improvement to an existing technology. For example, the fact that CNTs produce heat when irradiated with near-IR light means that they could be used for selective killing of cancer cells. However gold nanoshells show the same behaviour and are simpler to purify, characterise, handle, and process. Similarly, there are many cases of CNTs decorated with polymers and/or targeting moieties and associated with therapeutic molecules,[9, 11] but the primary rationale behind use of CNTs is either due to hydrophobic/π-stacking encapsulation or exploitation of high aspect ratio, both of which could be achieved using other methods. Lastly, there have been reports of *in vitro* and *in vivo* biodegradation of functionalized CNTs.[12] However, the kinetics of the degradative processes may be too slow to alleviate the risk from tissue accumulation from repeated, long-term use of CNT as a component of a therapeutic agent.

Given the challenges of CNTs delineated above, it is proving difficult to justify taking these materials forward into the clinic. However, we believe that these challenges are not the end of the story, but that they must be outweighed by the genuinely new properties introduced by the CNTs: a viable carbon nanotube biotechnology is one in which the unique properties of nanotubes bring about an effect that would be otherwise impossible. In this Outlook, we therefore seek to reframe the field by highlighting those biological applications in which the singular properties of CNTs provide some entirely new activity or biological effect, differing by type rather than just degree from existing technologies. We will not feature examples which do not fit this description, for which comprehensive reviews can be found elsewhere.[9, 11]

One-Dimensional Conductivity: Neural Interfaces

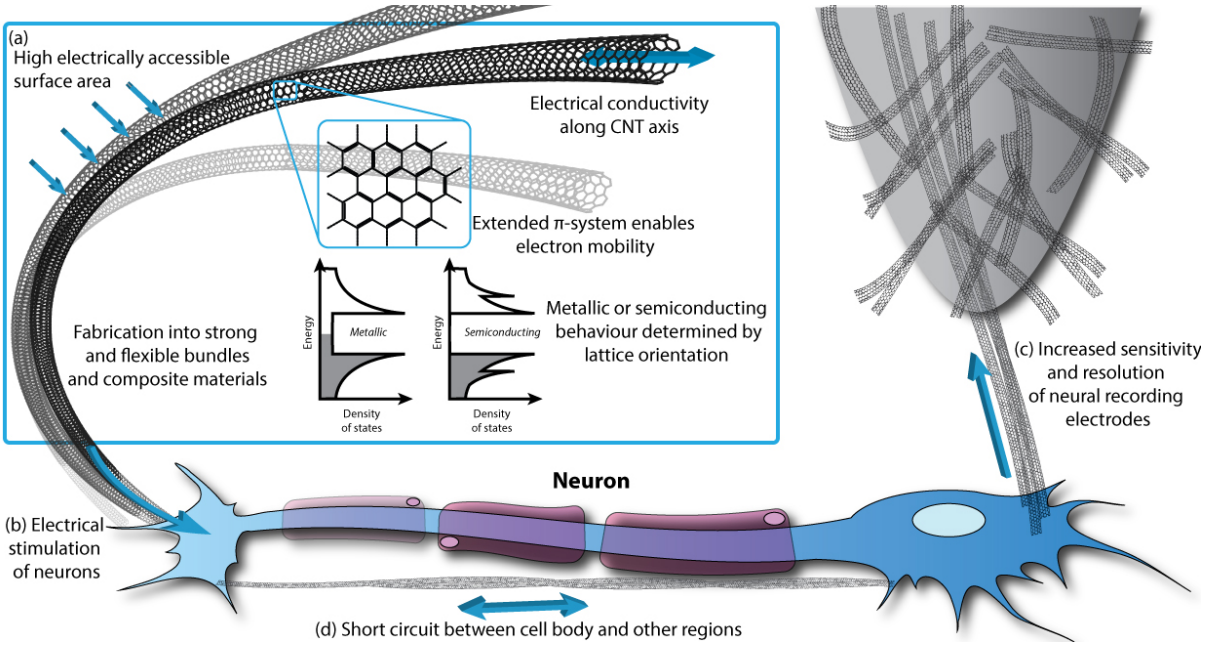

Figure 1. *(a) Important aspects of CNTs for use in neural interfaces. Illustration of using CNTs to (b) provide electrical input, (c) obtain output, and (d) alter neuronal behaviour.*

Box 1. *CNTs and neural interfaces: key points*

What is special about CNTs? Metallic CNTs are highly conductive 1D nanowires with a singularly high surface area, aspect ratio, and tensile strength.

What is the biological interest? Nature's electrical networks of neurons possess the secrets of sense, thought, movement, and memory, yet reading or influencing the system at cellular resolution is a major challenge.

What has been achieved? Ultrafine wires comprised of CNTs provide not only high resolution neuronal recording and stimulation, but can also induce new types of nervous activity and allow electrical cross-talk between muscle cells.

What does the future hold? Neuronal stimulation can be used to treat epilepsy and Parkinsons; being so thin, CNT electrodes for this purpose will produce far lower levels of inflammation. Further interface with muscular and sensory cells could help patients recover other functions.

As one-dimensional wires, CNTs display many advantageous properties for use in neural interfaces (Fig. 1a). The conductive properties of CNTs arise from the conjugated π-electron systems which extend over the entire structure resulting in either a continuum of electron energy levels, giving highly conducting CNTs, or a small gap, yielding semiconducting CNTs. CNTs also have an enormous electrochemically accessible surface area, maximising their prospects for interface. Their structurally anisotropic conductivity has a certain similarity to that of neurons. In fact, small CNT bundles have dimensions similar to those of dendrites (the branched protrusions of neurons), highlighting possibilities for integration. CNTs therefore can potentially provide a high-resolution solution for the readout, repair, or reconfiguration of neural networks in the central (brain, spinal cord) or peripheral nervous system.[13] No other technology has yet been described to offer such electronic control at such small length scales and with such control over directionality – metallic nanowires are at least an order of magnitude wider than the smallest CNT bundles used as electrodes, and do not display such anisotropy, nor proportional strength and rigidity.

Electrical stimulation of the nervous system can attain significant improvement of certain symptomatic effects in conditions such as epilepsy and Parkinsons.[14] Motivated by this fact, CNT fibre electrodes have been created for miniaturisation of both *in vivo* recording and stimulation of mouse brain tissue.[15] By making electrodes with a cross-section ten times smaller than that of the smallest metallic equivalent, the inflammatory response was significantly reduced.

On a different but related direction, studies by Bellerini *et al* [16] indicated that growth of neurons on surfaces decorated with MWCNTs resulted in a six-fold increase in the frequency of spontaneous action potentials, which are indicative of networked cells. Overall neuronal growth was not significantly different from samples prepared in the absence of CNTs. Although the mechanism for the improvement could not be elucidated, it was thought likely that the conductivity of CNTs was responsible. Soon afterwards, Kotov showed that neurons could be cultured on a CNT-film, differentiating naturally.[17] By applying a current laterally across the film, neuronal stimulation was achieved resulting in action potentials with a natural electrical current signature, confirming that CNT conductivity was involved (Fig. 1b).

Instead of using CNTs for electrical input, Keefer and coworkers used CNTs to output a high quality recording of neural activity by coating conventional metal wire electrodes with CNTs (Fig. 1c). In order to obtain adequate signal-to-noise ratio, the impedance of the electrode must be minimised, which usually requires tip enlargement. This increases the geometric surface area and thus reducing spatial resolution. By coating the tip with intrinsically high surface area CNTs they were able to increase the electrochemical surface area without compromising the overall tip diameter. The higher electrochemically accessible surface area was credited with a significant reduction in impedance, and hence higher contrast and time resolution in cultured neurons and even in rat motor cortex and primate visual cortex models.[18] Kotov and co-workers then developed electrodes composed of a layers CNT-polymer composite which were biocompatible and capable of differentiating biological signal of living mouse brains from instrument noise.[19] In 2015 Pasquali employed electrodes made of wet-spun CNT fibres[20] as neural recording probes.[15] The fibres had tissue contact impedances up to 20 times lower than that of PtIr and 6 times lower than tungsten wire of the same size. The lower impedance was attributed to the high ion-accessible surface due to interstitial spaces in CNT bundles. After surgical insertion into mouse brains, the fibres were more well tolerated than PtIr electrodes, while displaying the same neural recording capability.

Furthermore, Ballerini showed that CNTs could enhance and introduce new functions to neurons.[21] Rat hippocampal cells were cultured on a CNT film, and in addition to the increase in post-synaptic current frequency, they investigated the propensities of CNTs to assist in electrically-simulating the regenerative properties of neurons. Regular pulses were applied to the neuronal soma (main cell body), forcing action potentials, and somatic after-depolarisations (ADPs) were recorded. ADPs are indicative of Ca^{2+} electrogenesis in the peripheral dendrites of the cell mediated by potentials flowing in reverse, and require communication between the soma and the dendrites. ADPs were far more prevalent in cells cultured on CNTs than in those on glass, and the effect was consistent with CNTs acting as shortcuts between proximal and distal compartments of the neurons (Fig. 1d). Significantly,

neither using indium tin oxide which is highly conductive but smooth, nor insulating self-assembled peptide nanofibres of similar dimensions to CNTs, were the ADPs observed, indicating that the effect was particular to CNTs.

The next step for this technology is to interface CNTs with other electrically active cells. The first steps in this direction have been taken by Khademhosseini who has integrated CNTs into a crosslinked gelatin matrix for growth of cardiac muscle cells.[22] The resultant network of CNTs provides both mechanical strength and electrical conductivity to the material which mimics the extracellular matrix of the heart, and in particular is reminiscent of the conducting purkinje fibres which facilitate intercellular communication to give synchronous beating. Not only did the cells grow more uniformly on the CNT gel compared to the CNT-free analogue, but the spontaneous beating was more frequent and could be externally modulated through application of an electric field. It was even possible to release portions of the cell/gel film to give macroscopic beating bioactuators capable of swimming and pumping.

Overall, these studies have shown that there is scope for CNTs to be used to control and modulate neurons, to which they bear a certain similarity, being elongated and electrically active. The results garnered from the research above raise the possibility of using CNTs to gain insights into neuronal function. It is likely that through these techniques, the understanding of micro- and nanoscale neural phenomena will develop concurrently with the creation of CNT materials for neural stimulation and control. While these results are of great interest, there is a significant theoretical gap in terms of exactly how neurons and CNTs interact which requires further in-depth experimentation, knowledge that will be vital if these technologies are to be fully realised. Moving forward, an important goal is the interface of CNTs with other electrically active cells such as other forms of cardiac, muscle and sensory receptor cells. It can be hoped that such advances will help muscular regeneration and amelioration of impaired sensory input.

Versatile and Robust Encapsulation: Delivery of Undeliverables

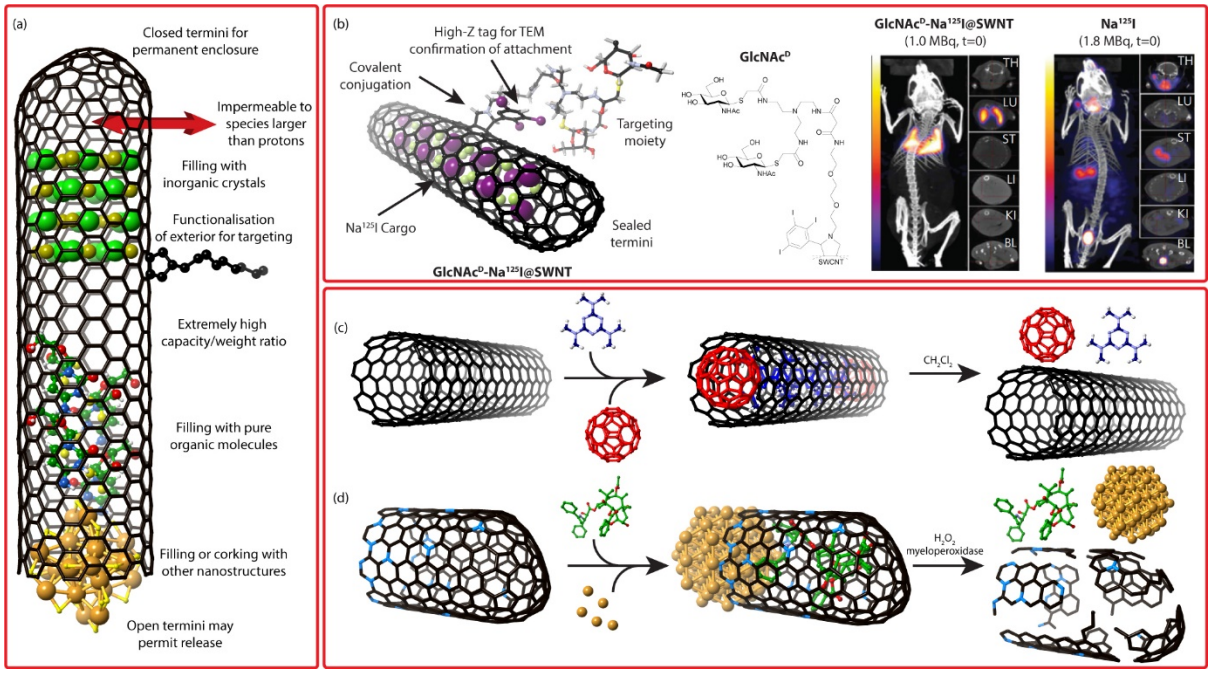

Figure 2. *Encapsulation in CNTs for biological applications. (a) Beneficial properties of CNTs for encapsulation. (b) $Na^{125}I$-containing nanocapsules decorated with glycosyl units, and SPECT images of mice treated with different doses of $Na^{125}I$@CNT and free $Na^{125}I$ showing characteristic biodistribution. Adapted by permission from Macmillan Publishers Ltd: Nature Mater.,9, 485-490, copyright 2010. (c) Filling CNTs with HMM and reversible sealing using C_{60}. (d) Filling of nitrogen-doped CNT cups with paclitaxel, corking with gold nanoparticles, and enzymatic digestion.*

CNTs are structurally hollow nanofibres, and this structural characteristic makes them attractive candidates for delivery of drugs and diagnostic agents (Fig. 2a).[23] Encapsulation of therapeutic agents within nanoscale constructs is a popular strategy for controlling biodistribution, ensuring that off-target effects are minimised. In the context of imaging, this approach gives maximal contrast. There are two important aspects in which CNT inclusion complexes differ from alternative encapsulation methods (e.g. polymer micelles, mesoporous silica, etc.). Firstly, the cavity is continuous, empty, and the bulk of nanotube's volume, meaning that anything from elemental metals,[24] ionic salts,[25] molecules,[26] or nanostructures[27] can be included in high yield. This contrasts especially with micellar architectures in which the host is a microphase in which the target molecule is soluble: with CNTs, the material itself can be encapsulated as a pure phase in itself. Secondly, the graphene shell can perfectly separate the core from the exterior environment, completely sealing the cargo if the ends are closed. The graphene lattice has been described as a perfect 'nanoballoon' [28], with prohibitively high energy barriers for translocation of atoms.[29] To illustrate this power, ionic solids which would be too soluble as nanoparticles, can be enclosed irreversibly within sealed CNTs by capillary force-mediated filling of CNTs in the melt, after which the ends spontaneously close upon cooling.[24, 30] Although a range of biological applications have been attempted for filled CNTs, only a few exploit the potential for sealing the cargo.

Box 2. *Filling and sealing CNTs: key points*

What is special about CNTs? The interior of CNTs can be filled with large quantities of almost any other chemical in its pure form. Both robust sealing and reversible corking are possible.

What is the biological interest? Permanent sealing can render otherwise incompatible substances suitable for biological media. Stimulus responsive corking could be used to create potent drug delivery vehicles which release their cargo only at the desired target.

What has been achieved? Mutual protection has been afforded to both biological systems and the nanotube cargo delivered; targeted capture-release of therapeutic molecules has been demonstrated up to the level of *in vitro* cellular studies.

What does the future hold? There is great promise for directed delivery of otherwise undeliverable therapeutics and imaging agents. Rigorous codification of production and purification protocols and extensive safety tests are required to make these approaches suitable for the clinic.

In collaboration with a number of other groups, we sealed radioactive $Na^{125}I$ inside SWCNTs to create a targeted imaging agent with no leakage of radioisotopes (Fig. 2b).[31] The $Na^{125}I$@SWCNTs were decorated with dendritic *N*-acetylglucosamine, $GlcNAc^{D}$ (known to be important in cellular recognition) using dipolar cycloaddition reactions with azomethine ylids. The resulting capsules were administered to mice and using single-photon emission computed tomography (SPECT), 3D tomograms of the biodistribution of $Na^{125}I$-filled CNTs, and free $Na^{125}I$ were compared. While the free $Na^{125}I$ was concentrated mainly in the thyroid and in the stomach, it was found that the CNT-encapsulated iodide was found almost entirely in the lungs, demonstrating that it was possible to override the prevalent uptake routes. Furthermore, it was possible to reduce the minimum visualisable dosage of radiation from 1.8 MBq (using free $Na^{125}I$) to just 0.2 MBq. This broke all previous records for radionuclide-based imaging, due to the intense concentration of iodide within the nanotubes and its stable retention.

Conversely, instead of protecting the organism from the cargo, Bonifazi and co-workers have used MWCNTs to shield a magnetic mixture of metallic iron and iron carbide from biological environments in which it would be unstable.[32] The CNTs were functionalised with an antibody against the epidermal growth factor receptor (EGFR) which is over-expressed in cancer cells. The Fe@MWCNTs were then used to magnetically separate cancer cells from a mixture of cells, providing a useful analytical tool and demonstrating their selectivity. Hyperthermia could then be induced under electromagnetic irradiation resulting in the death of only the EGFR-expressing cells. Although magnetic manipulation of biological matter using nanoparticles is known,[33] this is an elegant demonstration of the partitioning power of CNT walls.

Covalent sealing of nanotube caps requires vacuum annealing from 800°C,[34] limiting the number of usable substrates. Lower temperature strategies have therefore been proposed to keep material trapped within CNTs. Some report that materials can be stably included without additional engineering.[26] However, with such structures it is quite probable that low levels of leaching are still occurring. Pastorin *et al* [27a] have pioneered a method in which nanostructures are used as 'corks' to prevent leakage of the encapsulated material (Fig. 2c). They initially used C_{60} fullerenes (which have a high affinity for the CNT interior) to stopper CNTs filled with hexamethylmelamine (HMM, an anticancer drug). The HMM was

then inaccessible until washing with dichloromethane removed the corks and liberated the drug. Green took this further by appending amines to the C_{60}, resulting in release in aqueous acid, conditions relevant for cancer treatment.[35] Pastorin performed the first cell studies on corked CNTs using a *cis*-platin@MWCNT system stoppered with gold nanoparticles, however toxicity results showed that slow release still occurred from the supposedly plugged tubes.[27b]

A different approach to prevent escape of encapsulated matter has been reported by Bianco, Gazeau, and Bégin in which two rounds of magnetic ferrite nanoparticle synthesis within MWCNTs are performed, resulting in a 'jammed' system from which the nanoparticles cannot leave.[36] Magnetic manipulation was then used to enhance CNT uptake in ovarian cancer cells and move or agitate the cells themselves. A satisfyingly successful strategy has now been reported by Star, in which gold nanoparticles are grown on the rim of nitrogen-doped CNT cups, permitting sealing of drugs such as paclitaxel within (Fig. 2d).[37] Because of the doping, the CNT cups are highly susceptible to enzymatic oxidative degradation (which is known, but slow with pristine CNTs[38]), permitting their programmed disintegration in the presence of myeloperoxidase and hydrogen peroxide. Myeloid-derived suppressor cells, responsible for immunosuppressive response in cancer escape, overexpress this protein and was found that the paclitaxel payload was efficaciously delivered to these cells, promoting their differentiation into dendritic cells and cancelling their immunosuppressive character.

There is enormous scope for further work in this field. An extremely wide selection of materials can be sealed in CNTs which could interact with biological matter or imaging equipment in a non-contact fashion through the whole spectrum of electromagnetic processes. For example, no radionuclides have yet been sealed in CNTs for targeted therapy, and the crystalline nature of the cargo has not been exploited at all. Furthermore, although some optimisation of corking/uncorking may be required, there is also much potential for utilisation of the unique CNT hollow cavity for controlled capture-release under a variety of stimuli. In either case, the powerful demonstrations of orthogonalisation between payload and biodistribution showcase what may be possible.

Biological Barrier Translocators: from Molecular Transporters to Synthetic Membrane Channels

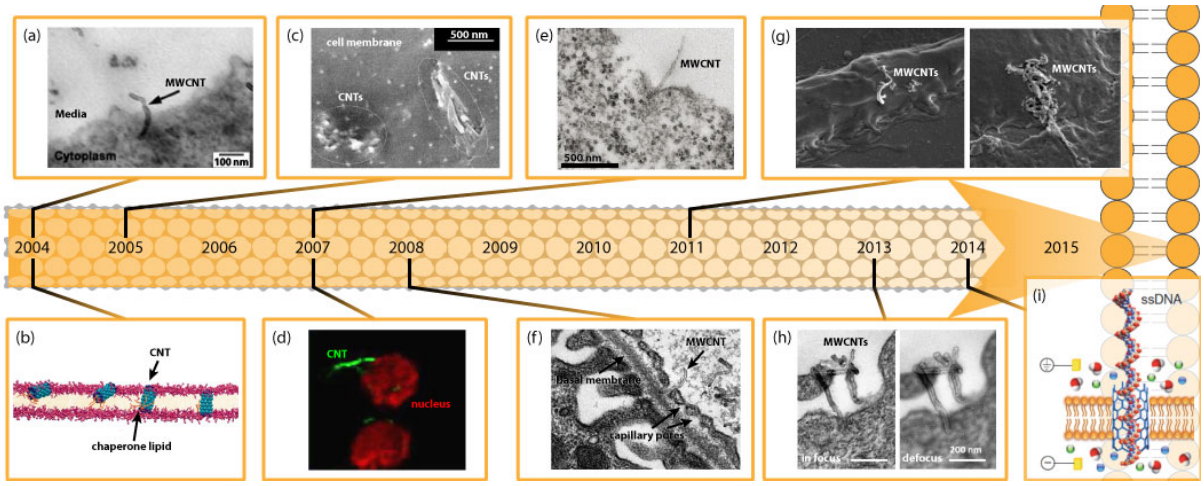

Figure 3. *Direct penetration of biological membranes by CNTs. (a) TEM image of ammonium-decorated MWCNTs entering HeLa cells.[39] (b) Molecular dynamics simulation of short SWCNT forming a membrane channel.[40] (c) SEM image of magnetic nanotube spearing of MCF-7 cells.[41] (d) Confocal fluorescence microscopy image of B-lymphocytes magnetically speared with CNTs.[42] (e) TEM image of a MWCNT penetrating a microglia cell.[43] (f) TEM image of individual MWCNTs passing through biological filters in the kidney.[44] (g) SEM images of MWCNTs crossing neuronal membranes.[45] (h) TEM images of ammonium-decorated MWCNTs crossing A549 cell membranes. (i) CNTs acting as membrane pores for translocation of DNA.[46]*

Box 3. *CNTs and cell membranes: key points*

What is special about CNTs? Nanotubes are widely documented directly piercing biological membranes, providing intimate access to the cell interior.

What is the biological interest? Cell uptake of nanomaterials most commonly occurs with the particles incarcerated within an inner membrane, thus hindering interaction with cellular function. Circumnavigation of this route is highly desirable.

What has been achieved? New methods for gene delivery, including a potential treatment for stroke.

What does the future hold? Once the specifics and scope of the phenomenon are established, we can expect go beyond a host of new drug delivery systems, towards direct interaction with the cellular interior.

Both experimental and theoretical studies of the last ten years have led to the understanding that short, surface-modified CNTs readily interact with biological membranes, other biological barriers and their constitutive components. The transport of cargo directly across biological membranes into the cell cytoplasm without causing damage is a major endeavour, as uptake of potentially therapeutic moieties is frequently restricted to membrane-bound targets. Such reported interactions of CNTs with biological membranes is a result of a unique combination of factors offered by CNTs: a) nanoscale fibre shape; b) the amphiphilic (hydrophobic-hydrophilic) surface character of chemically functionalised CNT; c) their range in dimensions (both longitudinal and lateral). All of these have led to unexpected and somewhat paradoxical observations at the back of which CNTs of certain design specifications are being developed as transporters of biologically active molecules and as artificial ion channels or their blockers.

The first direct observation of CNTs crossing cell membranes was reported in 2004 by Prato, Kostarelos, and Bianco, as part of a study using ammonium-functionalised CNTs as

transfection agents for delivery of externally complexed DNA.[39] MWCNTs could be seen in the process of crossing the membrane in a lengthwise manner (Fig. 3a), precluding an endocytotic process being involved. Accordingly, the complexed DNA was expressed in the cells. At around the same time, a theoretical study was put forth by Lopez et al in which short CNTs with hydrophilic end groups were found to spontaneously interact with lipid membranes.[40] Interestingly, partial immersion of the tube within the lipid bilayer was accompanied by crossing of individual lipid chaperone molecules (Fig. 3b). The study highlighted that the surface chemistry of the CNT was central to the behaviour – fully hydrophobic CNTs failed to form transmembrane channels.

Soon afterwards, fine control over delivery of CNTs (and respective cargo) into cells was documented by Cai and co-workers. By utilising CNTs which possessed ferromagnetic nickel particles at one end, they were able to create “nanotube spears.” By applying a rotating magnetic field, a swarm of rapidly moving CNTs was generated which was capable of penetrating cell membranes (as observed by SEM, Fig. 3c). By attaching DNA to the CNTs, it was possible to transfect a range of mammalian cell lines. Nanotube spearing of B lymphocytes was observed using phase contrast and confocal microscopy (Fig. 3d).[42] The process did not result in activation of the naïve B cells, which is vital for the study of these cells which are involved in malignancies and autoimmune diseases. Although in these cases, it was found that magnetic manipulation was necessary to achieve delivery of the DNA, seemingly in contradiction of the spontaneous direct penetration model, it should be noted that far lower concentrations of CNTs were used compared to other studies.

On the back of these original observations, the use of CNTs for treatment of disease conditions relating to the brain started to appear. The blood-brain barrier is a major hurdle for medicine, and if the unique penetration properties of CNTs could be harnessed in this area, it would be a significant contribution to healthcare. In 2007 it was reported that microglia, immune cells of the central nervous system (CNS) spontaneously take up polymer-dispersed MWCNTs.[43] Microscopy confirmed that direct membrane penetration was taking place (Fig. 3e), although variation in uptake between cell types suggested that other processes may occur concurrently. Impressive *in vivo* demonstration of the therapeutic potential of CNTs in the brain was provided in 2011 by Kostarelos and Pizzorusso.[45] Positively charged MWCNTs were used to complex siRNA and deliver it to rat brain cells. The RNA used interferes with the expression of the Caspase-3 protein which regulates cell death in reaction to stroke, leading to neuroprotective effects by preventing its release. The effective delivery of siRNA through neuron membranes was evidenced at the molecular level by reduction in Caspase-3 production *in vitro*. After direct injection into the cortex, internalisation of the CNTs within neuronal cells was clearly seen using electron microscopy (Fig. 3g). Most importantly, functional recovery was shown by improved performance of rats in a “skilled reaching” test, following induced stroke.

The interaction between CNTs and other biological barriers have also been elucidated. The appearance of CNTs in the urine of animals treated with the materials is now understood. Most nanomaterials are not excreted in this manner because renal clearance requires passage through pores in capillary walls (30 nm wide) followed by diffusion through a biopolymer matrix (200 – 400 nm long) and emergence between cellular filtration slits (40 nm wide). During chemical manipulation, CNTs are commonly collected on synthetic

membranes of much larger pores, therefore their passage into the urine was puzzling. Microscopy studies revealed that the MWCNTs with diameters of 20-30 nm were capable of reorientation *in vivo*, passing through the pores in a perpendicular manner (Fig. 3f).[44] The degree of individualisation, shape, and surface character of the CNTs involved are all expected to be central to this process. Further insights into the process of membrane binding and piercing were reported in a joint experimental-theoretical study in 2013, re-confirming that needle-like direct insertion into membranes is possible (Fig. 3h), and indicating that it is preceded by a complementary charge-mediated binding step.[8b] It should be noted that other, endocytosis-mediated uptake mechanisms have also been observed.[47]

In 2014 an important advance was reported by Geng and co-workers, providing a completely new mechanism for delivery through the cell membrane.[46] Very short (5-15 nm) lipid-coated, open-ended CNTs were shown to insert perpendicularly into lipid vesicles, creating a channel analogous to protein pore molecules. Transport of ions, water, and DNA through the channel into lipid membrane spheres was shown using single molecule measurements (Fig. 3i). Analogous measurements performed on live mammalian cells showed the same activity, indicating that it is possible to use CNTs to create a pipeline directly into the cell.

The ability of CNTs to directly cross the threshold between a living cell and its surrounding media is potentially a game-changing technology – no other method appears to show the same efficacy in bypassing the endosomal route. If it can be shown that they can traverse the blood-brain barrier, with a payload in tow, then a huge range of therapeutic possibilities await. However, although this direct piercing of membranes has been observed across many groups and types of CNT, it remains poorly understood. The ability to predict exactly what type of CNT, and with what surface chemistry, will traverse cellular membranes in each experiment is urgently needed. Moreover, the ability to create channels directly into cells opens up possibilities for direct and programmed communication with the cell interior.

Optical Absorption and Emission, Energy Transfer, and Semi-Conductivity: Photobionics

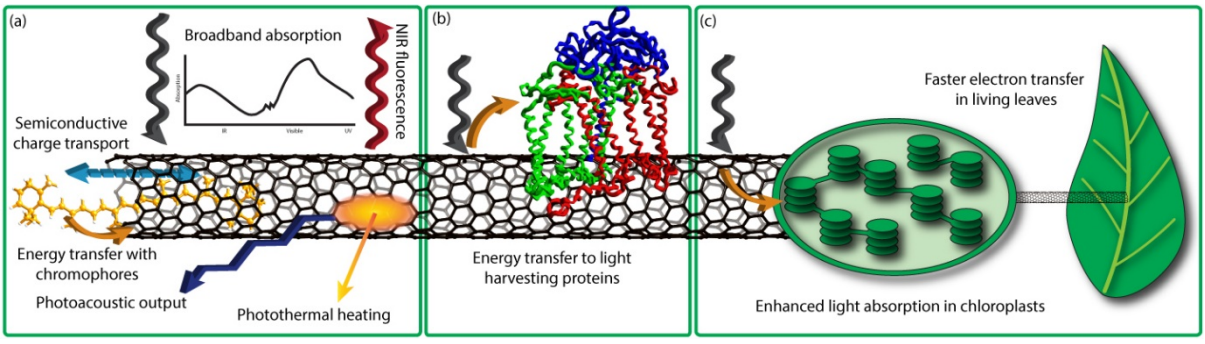

Figure 4. *(a) Optical properties of carbon nanotubes: the combination of broadband absorbance, energy transfer, and semiconductivity are vital for light harvesting applications, while fluorescent, mechanical and thermal outputs represent dead-end pathways. (b) Transfer of absorbed light to a photosynthetic centre protein. (c) Interface of CNTs with living photosynthetic machinery.*

Box 4. *Light harvesting with CNTs: key points*

What is special about CNTs? Nanotubes absorb an exceptionally wide spectrum of light and can transfer that energy over long distances to other species.

What is the biological interest? Optimising and harnessing the light harvesting photosynthetic machinery of plants (which does not use such a wide range of wavelengths) could lead to new biotechnological solar energy sources.

What has been achieved? The photosynthetic activity of plants has been increased by a factor of three through CNT infiltration.

What does the future hold? The extraction of directly usable power from enhanced plants would be a major achievement, leading to working bionic devices. Non-medical biotechnologies present a lower barrier to actualisation, opening up possibilities such as photobionic devices.

CNTs exhibit optical absorbance ranging from the UV to the far-IR.[48] In fact, forests of upright CNTs are currently the closest known material to a perfect black body absorber.[49] The energy of the absorbed light can be lost as heat, emitted as near-IR (NIR) fluorescence, or passed on to other species through energy transfer processes, meaning that CNTs can function as a highly receptive part of light harvesting cascades (Fig. 4a).[50] The potentially micron-scale exciton diffusion length of CNTs[51] makes them ideal for this purpose (although caution is required since disorder introduced even by non-covalent modification can curtail this significantly[52]). For example, encapsulated β-carotene can partake in energy transfer,[53] and the quenching of its photoluminescence by the CNT enables otherwise challenging vibrational spectroscopy to be performed on the molecule.[54] Given the growing desire for cheap and sustainable energy, the conversion of solar radiation into chemical energy is one of the present day's major challenges. However, the photosynthetic machinery within plants has been evolutionarily optimised not just for light harvesting on the molecular level, but for various aspects of fitness on the macroscopic scale, such as competition with other plants by shading.[55] Human use of photosynthesis is not subject to the same limitations, and would ideally seek to simply maximise the energy output, and for this reason there are strong grounds to investigate the artificial optimisation of photosynthesis.

In 2010 Strano and co-workers showed that photosynthetic reaction centres could be assembled reversibly into lipidic disks scaffolded along the length of SWCNTs (Fig. 4b). The resulting edifices showed a photocurrent which could be turned off upon disassembly.[56] However, although some perturbation of the CNT optical activities was observed, it was not conclusively shown that the CNT participated in energy transfer. In 2013, Bertoncini showed that light powered bacterial proton pump proteins take part in energy transfer when non-covalently attached to CNTs, with the mechanism depending on the pH.[57]

Strano then took the light harvesting capacity into living system using a plant nanobionics approach (Fig. 4c).[55] Semiconducting SWCNTs dispersed with charged polymers were inserted into extracted chloroplasts, and microscopy revealed that CNTs were localised in the chloroplast outer envelope. When infiltrated into plant leaves, the CNTs were again localised in the chloroplasts, greatly increasing the absorption bandwidth of their hosts. In the isolated chloroplasts, the photosynthetic activity was increased by a factor of three, while in leaves the rate of electron transport was also improved. Control experiments with purely

metallic CNTs gave no rate of improvement, indicating that the effect relied upon exciton mobility which occurs in semi-conducting materials.

The next big advances in this field will require the construction of bionic devices which can harness photosynthesis for generation of directly useable power. CNTs may play a major role here, both acting as photosensitisers, and energy conduits. As with neuronal interfaces, the details of interactions between the CNTs and the biological machinery require further study and further elucidation. However, these studies illustrate an important point: CNTs in biology can have applications beyond the realm of healthcare. Indeed, the exploitation of the life/CNT interface outside of the human body for purposes such as energy generation, waste processing, or novel materials, is a perhaps the ideal growth sector for CNT research in the short term for two reasons: the finer concerns for medical applications (which may not be settled for some time) are less important for energy science; and the interface with other non-natural materials is well developed, giving access to physical or chemical outputs. Nonetheless, there is also considerable potential for CNTs to interface with other biological photosensitive systems such as photoreceptor cells in the retina.

The optical absorption and energy-transfer properties of nanocarbon can also be used in other fields. Carbon nanohorns (tapered CNTs which tend to form spherical aggregates) have been used to induce NIR light-controlled muscular actuation in live frogs, through generation of reactive oxygen species (ROS) by attached dyes.[58] The presence of ROS stimulate ion pumps to produce an intracellular calcium flux, leading to a neuronal membrane current, and hence nervous signalling. Neither the dyes nor the nanohorns on their own resulted in stimulation, showing that the energy transfer process was integral. This leading example of remote cellular control opens doors to new tissue therapies and photobionic devices. Indeed, within the growing field of optogenetics[59] the NIR-activation of CNTs could be used much more broadly to influence cellular behaviour.

The emission properties of CNTs are yielding new opportunities in *in vivo* imaging systems. Animal tissue has substantial transparency in the two windows within the NIR spectral region, known as NIR-I (700-900 nm) and NIR-II (1200-1600 nm). Fluorophores with absorption and emission energies within these ranges have the potential to provide imaging modes with low background interference.[60] While there are an abundance of NIR-I fluorophores based on small organics, inorganic nanoparticles, and engineered proteins,[61] only CNTs provide fluorescence in the NIR-II region. Furthermore, quenching and decay of yield with time is negligible. In this longer-wavelength regime, reduced autofluorescence and scattering can provide greater penetration and resolution. The Dai group have developed proof-of-concept for this technique by coating SWCNTs with a lipid layer which disperses the CNTs in aqueous media without disrupted the side-wall chemistry necessary for fluorescence. Images of vasculature in living mice with resolutions of up to 35 μm and penetration beyond 2 mm were obtained without the need for prolonged exposure times.[62] However, since full 3D models of blood vessels (and other biological structures) with similar penetration, higher resolution, and similarly fast acquisition times can be obtained without contrast agents using NIR optical coherence tomography,[63] the strength of CNTs in NIR imaging lies in their ability to be targeted, and hence highlight otherwise invisible features. Using supramolecular decoration of CNTs with M13 viruses, Belcher and coworkers have been able to image both bacterial infections[64] and submillimetre tumours in mice,[65] with

subsequent guided surgery in the latter case. The next step for NIR-II imaging using CNTs must be to find and develop human-relevant applications in which the few-millimetres of penetration can be used to greatest effect.

Outlook

The cases discussed above illustrate that truly novel biological effects can be obtained through the use of CNTs, while also highlighting that such examples are still relatively few. Consolidation of effort and investment around these unique features and the applications that can be developed is needed for CNTs to be realised as components of widely used products.

This should provide impetus to conceive visionary CNT biotechnologies which will go beyond the optimisation of established strategies to the inception of new levels of potency in the biological domain.

Throughout the discovery and development of the technologies featured in this article, there has been considerable interest and discussion around the toxicological profile of CNTs. The perceived structural similarity with asbestos fibres seems likely to have been the main reason behind the concerns raised.[66] There have been numerous studies describing the risk of adverse reactions from exposure to CNTs (mainly SWNTs) using both cell and animal models and the mechanisms involved.[67] Most of this work has focused on the pulmonary route of exposure, with an emphasis on the occupational health risks associated with the production of nanotube material.[68] This important scientific discourse often does not reflect the parameters, conditions, material characteristics and levels of exposure that would be relevant in the context of a well-designed and highly regulated biomedical application and has somewhat unreasonably mired carbon nanotubes with scepticism regarding their safety profile. However, during the last 15 years of research in this area, critical conclusions have also been reached to indicate that: a) use of short (less than 1 µm long), adequately surface functionalized CNTs can alleviate almost entirely most of the risks associated with adverse reactions;[69] b) defect-rich (e.g. carboxylated)[70] or specifically surface-altered[71] CNTs can be made biodegradable. Importantly, the most recent opinion expressed by the International Agency for Research on Cancer (IARC; Lyon, France) following assessment of the potential carcinogenicity of carbon nanotubes (among other fiber-shaped materials) confirmed these findings.[72] Their conclusion was that the lack of coherent evidence across the various distinct types of CNTs precluded any (over)generalization. Only the long (13-20 µm long), rigid MWCNT-7 (Mitsui & Co.) was classified as possibly carcinogenic to humans (Group 2B classification), while SWCNTs and MWCNTs (excluding Mitsui-7 MWCNT) were categorised as ‘not classifiable’ as to their carcinogenicity to humans (Group 3 classification). It has to be stressed that no human cancer data were available to the IARC working group, indicating an inadequate overall body of evidence for the carcinogenicity of CNTs in humans. Nevertheless, such conclusive evidence and opinion hopefully will encourage the further development of well-designed CNT to utilize the unique properties described in the sections above.

There is enormous scope for exciting research and technology development in this area, as long as researchers have a clear idea of what unique benefit they expect to obtain through use of CNTs. In many cases this will occur through their combination of different properties, as seen above, while some of the more unique properties such as thermal conductivity, the

modulation of the properties of encapsulated species, and photoacoustic mechanical motion, remain unexplored in the biological arena. Conversely, there are areas of biology in which CNTs have not yet been employed to bring unique function, but could be useful, such as interface with biochemical reaction cascades, and cellular signalling and recognition. It is our opinion, especially for healthcare applications, that unless CNTs impart a unique property to the system, the challenges of multi-parametric polydispersity, and the cost of synthesis and purification will ultimately prove resultant technologies to be practically inexpedient. This should not discourage workers in the field, but rather provide impetus to conceive visionary CNT biotechnologies which will go beyond the optimisation of established strategies to the inception of new levels of potency in the biological domain.

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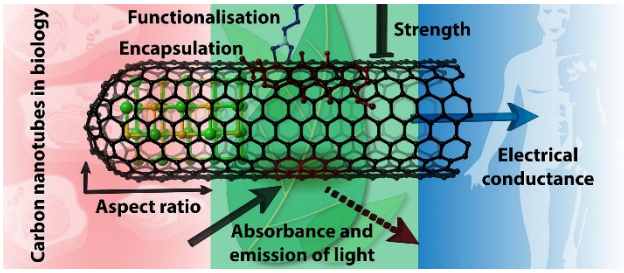

Carbon nanotubes display a huge variety of unique properties potentially valuable in biology, yet few are close to realisation. We assess the potential for nanotubes to yield unprecedented new applications through interface with life.